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neurotrophic growth factor, "enovin", an expression vector comprising said nucleic acid molecule, a host cell transformed with said vector, a neurotrophic growth factor encoded by said nucleic acid molecule, isolated enovin, compounds which act as agonists or antagonists of enovin and pharmaceutical compositions containing the nucleic acid or the enovin protein or the agonists or antagonists thereof.

Detailed Description of the Invention

According to a first aspect of the present invention there is provided a nucleic acid molecule encoding a human neurotrophic growth factor, designated herein as enovin, having the amino acid sequence illustrated in Figure 21, or encoding a functional equivalent, derivative or bioprecursor of said growth factor. Preferably, said nucleic acid molecule is DNA and even more preferably a cDNA molecule.

Preferably, the nucleic acid according to the invention comprises the sequence from positions 81 to 419 of the sequence illustrated in Figure 1 (SEQ ID No. 1) and more preferably from positions 81 to 422 and even more preferably the complete sequence illustrated in Figure 1 (SEQ ID NO. 2).

The nucleic acid molecule from position 81 to 419 (SEQ ID No. 1) is believed to encode the sequence of the mature enovin protein subsequent to processing of the proform of the protein at the RXXR processing site present in the stable proform of said enovin protein.

There is also provided by the invention an antisense molecule capable of hybridising to any of

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differentially spliced isoforms and transcriptional starts of the nucleic acids according to the invention.

In accordance with the present invention, a defined nucleic acid includes not only the identical nucleic acid but also any minor base variations including in particular, substitutions in bases which result in a synonymous codon (a different codon specifying the same amino acid residue) due to the degenerate code in conservative amino acid substitutions. The term "nucleic acid molecule" also includes the complementary sequence to any single stranded sequence given regarding base variations.

According to a further aspect the invention provides an isolated human neurotrophic growth factor, encoded by a nucleic acid molecule as defined herein. Preferably, the growth factor comprises an amino acid sequence from position 27 to 139 of the amino acid sequence of Figure 1 <u>(SEQ ID No. 3)</u> or a functional equivalent, derivative or bioprecursor thereof.

A "functional equivalent" as defined herein should be taken to mean a growth factor that exhibits all of the growth properties and functionality associated with the growth factor enovin. A "derivative" of enovin as defined herein is intended to include a polypeptide in which certain amino acids have been altered or deleted or replaced with other amino acids and which polypeptide retains the biological activity of enovin and/or which polypeptide can react with antibodies raised using enovin according to the invention as the challenging antigen.

Encompassed within the scope of the present invention are hybrid and modified forms of enovin,

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including fusion proteins and fragments. The hybrid and modified forms include, for example, when certain amino acids have been subjected to some modification or replacement, such as for example, by point mutation yet which modifications still result in a protein which retains the biological activity of enovin, according to the invention. Specific nucleic acid sequences can be altered by those of skill in the art to produce a growth factor exhibiting the same, or substantially the same, properties to enovin.

As is well known in the art, many proteins are produced *in vivo* with a (pre) signal sequence at the N-terminus of the protein. Furthermore, such proteins may comprise a further pro sequence that represents a stable precursor to the mature protein. Such pre and pro sequences are not generally necessary for biological activity. The enovin molecule according to the invention includes not only the full length sequence illustrated in Figure 21 but from position 27 to 139 (SEQ ID No. 3), which follows the RXXR proteolytic processing site present in growth factors of this type and which is believed to represent the mature sequence of enovin.

A defined protein, polypeptide or amino acid sequence according to the invention includes not only the identical amino acid sequence but isomers thereof in addition to minor amino acid variations from the natural amino acid sequence including conservative amino acid replacements (a replacement by an amino acid that is related in its side chains). Also included are amino acid sequences which vary from the natural amino acid but result in a polypeptide which is immunologically identical or similar to the

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an epitope thereof and recovering immune serum. Monoclonal antibodies may be prepared according to known techniques such as described by Kohler R. and Milstein C., Nature (1975) 256, 495-497.

Antibodies according to the invention may, advantageously, be used in a method of detecting for the presence of a growth factor according to the invention, which method comprises reacting the antibody with a sample and identifying any protein bound to said antibody. A kit is also provided for performing said method which comprises an antibody according to the invention and means for reacting the antibody with said sample.

Also provided by the present invention is a kit or device for detecting for the presence of a neurotrophic growth factor according to the invention in a sample, comprising an antibody as described above and means for reacting said antibody and said sample.

Proteins which interact with the neurotrophic factor of the invention, such as for example <u>its</u> [it's] corresponding cellular receptor may be identified by investigating protein-protein interactions using the two-hybrid vector system which is well known to molecular biologists (Fields & Song, Nature 340:245 1989). This technique is based on functional reconstitution in vivo of a transcription factor which activates a reporter gene. More particularly the technique comprises providing an appropriate host cell with a DNA construct comprising a reporter gene under the control of a promoter regulated by a transcription factor having a DNA binding domain and an activating domain, expressing in the host cell a first hybrid DNA sequence encoding a first fusion of a fragment or all

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gastrointestinal transit. Consequently a method of treatment is provided for relieving patients from conditions, such as, for example, gastrooesophageal reflux, dyspepsia, gastroparesis, post-operative ileus, and intestinal pseudo-obstruction.

Dyspepsia is an impairment of the function of digestion, that can arise as a symptom of a primary gastrointestinal dysfunction, especially a gastrointestinal dysfunction related to an increased muscle tone or as a complication due to other disorders such as appendicitis, galbladder disturbances, or malnutrition. Dyspeptic symptoms are for example a lack of appetite, feeling of fullness, early satiety, nausea, vomiting and bloating.

Gastroparesis can be brought about by an <u>abnormality</u> [abnormmaly] in the stomach or as a complication of diseases such as diabetes, progressive systemic sclerosis, anorexia, nervosa and myotonic dystrophy.

Post-operative ileus is an obstruction or a kinetic impairment in the intestine due to a disruption in muscle tone following surgery.

Intestinal pseudo-obstruction is a condition characterized by constipation, colicky pain, and vomiting, but without evidence of physical obstruction.

The compounds of the present invention can thus be used either to take away the actual cause of the condition or to alleviate the symptoms of the conditions.

Additionally some of the compounds being stimulators of kinetic activity on the colon, may be useful to normalize or to improve the intestinal transit in subjects suffering from symptoms related to

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disturbed motility, e.g. a decreased peristalsis of the small and large intestine alone or in combination with delayed gastric emptying.

In view of the colon kinetic utility of the compounds of the present invention, there is provided a method of treating warm-blooded animals, including humans, suffering from motility disorders of the intestinal system, such as, for example, constipation, pseudo-obstruction, intestinal atony, post-operative intestinal atony, irritable bowel syndrome (IBS), and drug-induced delayed transit.

Compounds identified as antagonists according to the assays of the present invention may also be of potential use in the treatment or prophylaxis of gastrointestinal conditions resulting from increased peristaltic movements in the intestines such as diarrhea (including secretory diarrhea, bacterial induced diarrhea, choleic diarrhea, traveller's diarrhea and psychogenic diarrhea), Crohn's disease, spastic colon, irritable bowel syndrome (IBS), <u>and</u> diarrheapredominant irritable bowel gastrointestinal hypersensitivity.

In view of the utility of the compounds of the invention, it follows that the present invention also provides a method of treating warm-blooded animals, including humans suffering from gastrointestinal conditions such as irritable bowel syndrome (IBS), especially the diarrhoea aspects of IBS. Consequently a method of treatment is provided for relieving patients suffering from conditions such as irritable bowel syndrom<u>e</u> (IBS), diarrheapredonminant irritable bowel syndrome; bowel hypersensitivity, and the reduction of pain associated with gastrointestinal

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are indicated in bold. A potential N-glycosylation site is double underlined[,].

Figure 2: is alignment of the predicted mature protein sequences of human GDNF, NTN, PSP and EVN. The sequences were aligned using the ClustalW alignment program. Amino acid residues conserved between all three proteins are included in the black areas. Residues conserved between two or three of the sequences are shaded in grey. The 7 conserved cysteine residues characteristic for members of the TGF-β family are indicated by asterisks above the sequence. Amino acid residues are numbered to the right. The dashes indicate gaps introduced into the sequence to optimize the alignment[,].

Figure 3: is partial cDNA sequence of enovin. The consensus sequence was obtained by PCR amplification (primary PCR with primers PNHsp1 and PNHap1 and nested PCR with primers PNHsp2 and PNHap2) on different cDNAs followed by cloning and sequence analysis and comparison of the obtained sequences. The translated one letter code amino acid sequence of nucleotides 30 to 284 (reading frame A) is shown above the sequence and numbered to the right (A1 to A85). This reading frame contains a putative ATG translation start codon. The translated one letter code amino acid sequence of nucleotides 334 to 810 (reading frame B) is shown above the sequence and numbered to the right (B1 to B159). This reading frame contains the region of homology with GDNF, NTN and PSP. The nucleotide residue number is shown to the right of the DNA sequence. The putative RXXR cleavage site for the

prodomain is indicated in bold and underlined. The putative start of the mature protein is indicated by an arrow. The seven conserved cysteine residues characteristic for all members of the TGF-β family are indicated in bold. A potential N-glycosylation site is double underlined[,].

Figure 4: is an illustration of the chromosomal localisation of human Enovin. (A) Diagram of FISH mapping results for Enovin. Each dot represents the double FISH signals detected on human chromosome 1, region p31.3-p32. (B) Example of FISH mapping of Enovin. The left panel shows the FISH signals on chromosome 1. The right panel shows the same mitotic figure stained with 4',6-diamidino-2-phenylindole to identify chromosome 1[,].

Figure 5. is an illustration of expression of Enovin in different human tissues. (A), (B), (C) Northern blot analysis of tissue expression of Enovin. The expression of Enovin mRNA in different human tissues was assessed using a probe corresponding to part of the coding region of Enovin (including the region coding for the mature Enovin protein) to analyse blots of human poly(A) rich RNA. (A) Multiple Tissue Northern (MTN) blot; (B) MTN blot II) Fetal MTN blot II. Panel (D) shows an autoradiography of the human RNA master blot probed with the same Enovin cDNA fragment. Panel (E) shows the location of human tissue mRNA samples on the RNA master blot from (D)[,].

Figure 6: is a graphic illustration of the total survival of SH-SY5Y cells after 72 hours treatment

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with 10-6M taxol and the effect of increasing doses of enovin on this survival, normalised to the condition of solvent. SH-SY5Y cells are differentiated for 5 days with 25nM staurosporine before application of taxol. Data are from two independent experiments in sixtuplate. Mean and st. dev. is shown[,].

Figure 7: is a graphic representation of the effect of increasing concentrations of enovin over 48 hours on neurite outgrowth of staurosporine - differentiated SH-SY5Y cells, normalised to the condition of solvent. SH-SY5Y cells are differentiated for 5 days with 25nM staurosporine before starting the 48 hour experiment. As a positive control, the differentiating effect of 25nM staurosporine is shown. Neurite length is calculated on at least 5000 cells. Data is provided from the experiments performed in duplicate. Mean and St. dev. is shown.

Figures 8 to 18: are graphic representations of the effect of enovin on proliferation of various cell types.

Figure 19: is a graphic representation of the effects of enovin on taxol-induced sensory deficits using the pin prick test. Given are the average (± 1 SEM) cumulative scores over time of rats treated with either 2 different doses of enovin (23 or 130 g/ml; n = 10 rats/ group) or vehicle / saline (n = 20 rats) after taxol. Enovin or saline / vehicle were injected in a volume of 75 l in the subplantar area of the right hind paw.

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sequence coding for a putative novel protein similar to the neurotrophic factors *GDNF, NTN* and PSP which has been called enovin (EVN). Additional database homology searching using the genomic DNA sequence surrounding the region coding for enovin yielded several expressed sequence tag (EST) clones derived from different human tissues (prostate epithelium [accession no. AA533512 (ID1322952)], lung carcinoma [accession no. AA931637] and parathyroid tumor [accession no. AA844072]). These clones contain DNA sequence outside of the region of homology with GDNF, PSP or NTN, but confirmed that enovin mRNA is expressed in normal and tumor tissues.

Initial PCR amplification using primers (PNHsp3 and PNHapl) based on the genomic sequence yielded a fragment of ≈ 500 bp from fetus, fetal brain, prostate, frontal cortex, hippocampus, cerebellum cDNA and from genomic DNA, but not from lung cDNA. Cloning and sequence analysis of these fragments yielded a DNA sequence of 474 bp that translated into a predicted protein sequence of 139 amino acid residues including seven conserved cysteine residues characteristic of all the members of the transforming growth factor β (TGF-β) family of proteins (Kingsley, 1994) (Figure 1) (SEQ ID Nos. 2 and 4). The sequence also contained a RXXR motif for cleavage of the prodomain (RAAR, amino acid position 23 to 26) (Barr, 1991) . A similar cleavage site is present in the GDNF, NTN and PSP protein sequences, at a comparable position in the prodomain sequence. Assuming cleavage of the enovin prodomain occurs at this site *in vivo*, the mature EVN protein sequence contains 113 amino acid residues (residue 27 to 139 in figure 1) (SEQ ID No. 3) and has a calculated molecular mass of 11965

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sequence of 819 bp (Figure 3) <u>(SEQ ID No. 5)</u>. This sequence contains a putative ATG start codon at nucleotide positions 30-32 and yields an open reading frame (reading frame A in figure 3) <u>(SEQ ID No. 6)</u> that extends up to a stop codon at nucleotide positions 285-287. The translated protein sequence of this region does not show similarity to any known protein in the databases. Translation of the cDNA sequence in the second reading frame (reading frame B in figure 3) <u>(SEQ ID No. 7)</u> yields a predicted protein sequence of 159 amino acid residues. This sequence contains the RXXR cleavage site (position B43 to B46; nucleotide position 460-471) and the sequence corresponding to the mature enovin sequence (position B47 to B159; nucleotide position 472-810). The open reading frame including the RXXR cleavage site and the mature enovin coding sequence extends from nucleotide position 334 (preceded-by an in-frame stop codon) to a stop codon at position 811-813, but does not *contain* an ATG codon for a starting methionine residue. In analogy with persephin (Milbrandt et al., 1998) we hypothesize that an unspliced *intron* is present in the majority of the mRNA transcripts from the EVN gene. GDNF and NTN also have an intron in their respective prodomain coding regions (Matsushita et al., 1997, Heuckeroth et al., 1997).

To evaluate the existence of different mRNA transcripts for Enovin, RT-PCR experiments were performed using primers situated at the 5' end of the Enovin coding sequence just 5' of a possible upstream ATG start codon (primer PNHsp5 [5'-GCA AGC TGC CTC AAC AGG AGG G-3'] and nested primer PNHsp6 [5'-GGT GGG GGA ACA GCT CAA CAA TGG-3'] and at the 3' end (primer PNHap1 and nested primer PNHap2 [see Table 1].

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Experiments were performed on human multiple tissue cDNA panels (Clontech MTC panels I and II), on a fetal heart cDNA library (Clontech) and on cDNA derived from human cerebellum, hippocampus or frontal cortex (Masure et al., 1998). Primary PCR reactions were performed with primers PNHsp5 and PNHap1 under GC-rich conditions (Advantage GC-PCR kit, Clontech) for 30 cycles (95°C - 30s, 60°C - 30s, 72°C - 1 min) as described. Nested PCR reactions were performed on 1 l of the primary PCR product using primers PNHsp6 and PNHap2 under the same conditions for 30 cycles. Resulting PCR products were analysed on a 1.5% agarose gel and ranged in size from ± 350 bp to ± 800 bp. Several bands were purified from the gel and the PCR fragments sequenced directly. Some purified PCR products were also cloned in vector pCR2.1-TOPO (TOPO-TA cloning kit, Invitrogen) and then sequenced. Sequence analysis confirmed the existence of different mRNA molecules containing Enovin sequence. The obtained fragment sequences were compared with the genomic Enovin sequence. This allowed us to identify several possible 5' and 3' splice sites in the genomic sequence (Figure 21)<u>(SEQ ID Nos. 11 through 15)</u>. All these splice sites corresponded to the consensus sequences for donor and acceptor splice sites (Senapathy, P., Shapiro, M.B. & Harris, N.L. (1990)) splice junctions, branch point sites, and exons: sequence statistics, identification, and applications to genome project. Methods Enzymol. 183,252-—278). The different Enovin splice variants identified and their presence in different human tissues are summarized in Figure 22. Only two of the 5 sequenced transcripts yield functional Enovin protein upon translation from the ATG start codon.

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These two transcripts code for proteins of 228 or 220 amino acids, respectively with predicted signal peptides of 47 and 39 amino acid residues. The predicted protein sequences of these two variants are shown in Figure 23 (long variant) (SEQ ID No. 9) and Figure 24 (short variant) (SEQ ID No. 10). The long variant can be deduced from the DNA sequence of Figure 21 (SEQ ID No. 8) by splicing out the first intron at locations 5'-1 and 3'-1 and the second intron at 5'-2 and 3'-3 (SEQ ID No. 12). Upon translation of the open reading frame, the predicted protein sequence of Figure 23 is obtained. The shorter variant can be deduced from the DNA sequence of Figure 21 by splicing out the first intron at locations 5'-1 and 3'-2 and the second intron at 5'-2 and 3'-3. Upon translation of the open reading frame, the predicted protein sequence of Figure 24 is obtained.

The longest transcript seems to be the most abundant in most tissues as judged by the band intensity in Figure 22B. The shorter transcripts result in frame shifts yielding a translated protein missing the mature Enovin amino acid sequence homologous with GDNF, NTN and PSP. The two smallest transcripts even miss part of the mature coding sequence, including two of the seven highly conserved cysteine residues. Figure 22B shows the distribution of the main splice variants in different human tissues. Functional Enovin mRNA is expressed in almost all tissues tested, including brain, heart, kidney, liver, lung, pancreas, skeletal muscle, colon, small intestine, peripheral blood leukocytes, spleen, thymus, prostate, testis, ovary, placenta and fetal heart. In some human tissues (e.g. cerebellum, hippocampus), only non-functional transcripts could be